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**DPP6A Confers Redox Sensitivity to Kv4 Channel Inactivation**

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Reactive oxygen species (ROS) are important modulators of excitability and may play a critical role in the etiology of many neurodegenerative disorders. Previous studies have shown that the fast N-type inactivation of Kv1.4 channels is suppressed by ROS-mediated cysteine oxidation whereas Kv4 channel N-type inactivation is not. However, in native neurons Kv4 channel subunits form large macromolecular complexes with KChIP and DPL proteins, and fast N-type inactivation is conferred to Kv4 channel complexes by a specific isoform of DPL proteins, either DPP10a or DPP6a. To investigate whether the DPP6a-mediated fast inactivation is regulated by ROS, tert- butyl hydroperoxide (tBHP) was applied to oocytes expressing Kv4.2+KChIP3a+DPP6a channels. tBHP (1 mM) application dramatically increases the peak current amplitude by ~ 44% and slowed inactivation kinetics. The effects of tBHP are reversed by the reducing agent dithiothreitol (DTT, 10 mM). In contrast, ternary complex channels containing another DPP6 isoform (DPP6K) are not affected by tBHP, indicating the importance of the DPP6a variable N-terminal domain for the tBHP effect. Alignment of N-terminal sequences from DPP6a and DPP10a with Kv1.4 reveals a common cysteine residue in position 13 (Cys-13), which in Kv1.4 is critical for the redox-regulation of N-type inactivation. Substituting Cys-13 of DPP6a with serine (DPP6a/C13S) results in a loss of regulation by tBHP, consistent with a similar critical role for DPP6a Cys-13. To test if other cysteines in the channel are also required for this regulation, we switched to mutant Kv4.1 (C11xA) and KChIP3a (KChIP3a/del2-59) constructs that remove most intracellular cysteine residues. Channels composed of C11xA, KChIP3a/del2-59, and normal DPP6a show disrupted regulation by tBHP, suggesting that ROS likely induces an intersubunit disulfide linkage to regulate DPP6a-mediated N-type inactivation. This work was supported by a grant from the National Institute of Health (R01 GM090029).

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**Ci-KCNQ1, an Ortholog of Vertebrate KCNQ1 from Ciona Intestinalis, has Revealed that KCNE1 and KCNE3 Utilize Different Domains of KCNQ1 for the Modulation of Gating**

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KCNQ1 channel is a voltage-gated potassium channel expressed in various tissues such as heart, intestine, inner ear, kidney and pancreas. The gating property of KCNQ1 is largely determined by the type of coexpressed KCNE proteins. For example, coexpression of KCNQ1 with KCNE1 produces slowly-activating potassium current which is known as $I_{Ks}$ in heart, while KCNE3 makes KCNQ1 voltage-independent and constitutively-active. We previously isolated Ci-KCNQ1, a vertebrate KCNQ1 ortholog from marine invertebrate *Ciona intestinalis*. As KCNE genes are not present in the *Ciona* genome, we hypothesized that Ci-KCNQ1 might lack important amino acid residues for the gating modulation by mammalian KCNE proteins. Ci-KCNQ1, by itself, produced large voltage-gated potassium current (about 10 μA at + 40mV) in *Xenopus* oocyte. As we hypothesized, KCNE proteins did not properly modulate Ci-KCNQ1 as they did with human KCNQ1. KCNE1 shifted the *G-V* curve to the negative direction and failed to transform Ci-KCNQ1 into a slowly-activating potassium channels. Ci-KCNQ1 remained voltage-dependent in the presence of KCNE3. By making series of chimeras of human KCNQ1 and Ci-KCNQ1, we identified that some amino acid residues in the pore region of Ci-KCNQ1 are responsible for the improper modulation with KCNE1. On the other hand, the difference of S1 segment seemed to be responsible for the lack of modulation by KCNE3. Point mutation revealed that Phe127 on the S1 segment of human KCNQ1, which is substituted with valine in Ci-KCNQ1, plays an important role in the KCNE3 modulation. Taken together, we showed that KCNE1 and KCNE3 utilize different domains of KCNQ1 channels, and that may be the reason why different KCNE proteins exert extremely different modulation effects on KCNQ1.

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**Competitive Interactions of Kv7 Channel Carboxy-Termini with $PIP_2$ and Calmodulin**

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Calmodulin (CaM) is a ubiquitous calcium sensor which binds to the carboxyl terminus of Kv7 channels, thus regulating channel function. $Ca^{2+}$-dependent interactions of Kv7 with CaM mediate channel inhibition by specific G protein coupled receptors (GPCR). Yet other GPCR's inhibit Kv7 channels via the depletion of membrane phosphatidylinositol 4,5-bisphosphate ($PIP_2$), which is required for Kv7 activity. Putative binding sites for $PIP_2$ and CaM are in close proximity or overlap within the proximal C-termini of Kv7 channels. We investigated whether calmodulin and $PIP_2$ binding to Kv7 channels is competitive, a phenomenon that would predict an increased affinity for Kv7-CaM interaction upon $PIP_2$ depletion, and, conversely a decrease of Kv7 channel $PIP_2$ affinity upon CaM binding. We performed co-immunoprecipitation between CaM and Kv7.4 overexpressed in HEKMSR cells under conditions of tonic $PIP_2$, chronic $PIP_2$ depletion (overexpression of $PIP_2$ sequestering construct) and chronic $PIP_2$ elevation (overexpression of phosphatidylinositol 5-kinase, PI5K). Sequestering $PIP_2$ increased the co-immunoprecipitation of CaM with KCNQ4, while overproduction of $PIP_2$ decreased CaM-binding. Next, we evaluated fluorescence recovery after photobleaching using TIRF illumination (TIRF-FRAP) between KCNQ4 and eYFP-CaM under conditions of normal, low and high membrane $PIP_2$. When only eYFP-CaM was expressed in HEKMSR cells, TIRF-FRAP had a time constant of $43 \pm 9$ s (n=24), co-expression of CaM with Kv7.4 showed an increase in the recovery time constant to $93 \pm 21$ s (n=24, $p \leq 0.05$), indicating a fraction of CaM molecules are tethered to the plasma membrane by the interaction with Kv7.4. Depletion of $PIP_2$ with wortmannin (10 μM) resulted in a further increase in recovery time to $453 \pm 165$ s (n=27, $p \leq 0.001$); overexpression of PI5K resulted in the recovery time of $118 \pm 19$ s (n=20, $p \leq 0.001$ vs. wortmannin). Collectively, these data strongly suggest a competitive nature for CaM and $PIP_2$ interactions at the Kv7 C-terminus.

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**The CRAC Motif Nearest to the N-End of Slo1 Cytosolic C-Tail is the Main Determinant of BK Channel Sensitivity to Membrane Cholesterol**

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Increased membrane cholesterol (CLR) decreases BK channel activity (Po) (reviewed by Levitan et al., 2010). Electrophysiological data from a recent structure-activity study of CLR and several analogs on BK channel-forming slo1 proteins (cbv1) reconstituted into phospholipid bilayers led us to hypothesize that CLR action resulted from CLR selective and direct recognition by a site(s) in cbv1 (Bukiya et al., 2011).

We identified ten Cholesterol Recognition/Interacting Amino acid Consensus (CRAC) domains in cbv1: CRACs 1-3 in the transmembrane core (S0-S6), and CRACs 4-10 in the cytosolic C tail (CTD). Bilayer electrophysiology demonstrated that construct trcbv1S6, which lacked CTD, failed to respond to CLR. However, an extended construct that included CRAC4 (trcbv1-CRAC4) was CLR-sensitive, indicating that a major determinant of CLR sensitivity lies in CRAC4 and/or the linker between this motif and S6. We next removed two CRACs at a time: immediately distal to CRAC6 (trcbv1-CRAC6) and to CRAC8 (trcbv1-CRAC8). Trcbv1-CRAC6 and trcbv1-CRAC8 were CLR-sensitive, further supporting the hypothesis that the S6-CRAC4 linker and/or CRAC4 itself is essential for CLR action. However, elimination of CRACs 7-10 or CRACs 9-10 somewhat decreased CLR action. To determine whether CTD CRACs themselves contributed to CLR responses, we introduced progressive Phe substitutions of signature tyrosines in CTD CRACs. The most drastic reduction of CLR action occurred with Y450F in CRAC4. However, cumulative Tyr to Phe substitutions in CRACs 5-10 gradually reduced CLR action. In conclusion: 1) CRAC4 in BK CTD is the main region contributing to CLR action; 2) the signature CRAC residue Y450 is essential for CLR sensitivity; 3) tyrosines in CRACs distal to CRAC4 also contribute to overall CLR sensitivity of BK channels. Support: R01-HL104631;R37-AA011560 (AMD); UTHSC NI Fellowship (AKS).

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**Sodium 3-Hydroxyolean-12-en-30-Oate is a Novel and Selective Activator of β1 Subunit-Containing BK Channels and thus Cerebral Artery Dilator**

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Lithocholic acid (LCA) is a cholane steroid that causes cerebrovascular dilation *via* activation of β1 subunit-containing BK channels in vascular smooth muscle (Bukiya et al., 2007; 2009). In search of selective β1-containing BK activators devoid of a steroidal nucleus, we used LCA as a template and performed structure similarity search with the threshold of 70% in the Hit2lead.com database. We identified methyl 3-hydroxyolean-12-en-30-oate, which after hydrolysis, rendered sodium 3-hydroxyolean-12-en-30-oate (HENA). Patch-clamp results demonstrated that HENA activated BK channels (cbv1+β1) cloned from rat cerebral artery myocytes. While $EC_{50}$ (~50 μM), $E_{max}$ (~300 μM) and apparent

Hill number (1.3) for this HENA action were undistinguishable from those of LCA, HENA's efficacy was significantly larger than that of LCA, with channel steady-state activity in presence of 150 μM HENA increasing to 200% of pre-drug values. Remarkably, HENA failed to activate recombinant, β2-, β3-, or β4-containing BK channels while activating native BK channels in rat cerebrovascular myocytes ($EC_{50}$=46 μM). Therefore, HENA selectively targets β1-containing BK channels. Furthermore, HENA failed to activate cbv1+β1T169A channels, suggesting it acts *via* a BK β1 cholane-sensing site (Bukiya et al., 2008). HENA (3-45 μM) dilated pressurized cerebral arteries of rat and C57BL/6 mouse. Consistent with its higher efficacy on channel activity, HENA-induced dilation was significantly larger than that of LCA. As expected, genetic ablation of BK β1 suppressed HENA-induced vasodilation. Finally, closed cranial window data from anesthesized rats demonstrated that intracarotid infusion of HENA significantly dilated pial arterioles. This action was sustained in presence of 4-aminopyridine but totally prevented by paxilline, underscoring BK-mediation of HENA-induced *in vivo* vasodilation. This study identifies the first selective non-steroid activator of β1-containing BKs and effective cerebral artery dilator. Supported by R01-HL104631; R37-AA011560 (AMD).

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**Beta Subunit Transmembrane Domains Determine the Differential Ethanol Responses of β1- vs. β4-Containing BK Channels**

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Ethanol (10-100 mM) usually decreases the steady-state activity (Po) of vascular smooth muscle BK channels while increasing Po of neuronal BK channels (Brodie et al., 2007). Native BK complexes consist of channel-forming α and tissue-specific, accessory β subunits. Four β subunit types were identified, with β1 and β4 prevailing in smooth muscle and neurons, respectively (Brenner et al., 2000). Remarkably, differential expression of β1- vs. β4-containing BK channels modifies Po adaptation to protracted ethanol exposure (Feinberg-Zadek et al., 2008; Martin et al., 2008). On the other hand, β1 is necessary for ethanol to inhibit vascular smooth muscle BK channels and the resulting vasoconstriction (Bukiya et al., 2009). To begin to address the mechanisms and protein regions contributing to differential ethanol actions on BK channels, we expressed channel-forming cbv1 subunits in absence and presence of β1 or β4. Then, we conducted $G/G_{max}$-V plots from ionic currents in inside-out patches and evaluated their modification by acute ethanol exposure. As described for other BK channels (Orio et al., 2002), β1 but not β4 significantly reduced cbv1 V0.5 across 1-1,000 μM calcium. Ethanol (50 mM) decreased homomeric cbv1 V0.5 at low calcium while increasing V0.5 at high calcium, the "crossover" from current potentiation to inhibition occurring at 20 μM calcium. This crossover was left-shifted to 2 μM by β1 co-expression but unaltered by β4 co-expression. Therefore, β1 enables ethanol inhibition of current at calcium levels that are reached near the BK channel in myocytes. Evaluation of $G/G_{max}$-V plots from currents mediated by cbv1 ± chimeric β1/β4 demonstrated that ethanol responses of complexes containing β chimeras that included β1 TM domains mimicked cbv1+*wt*β1 responses. Conversely, ethanol responses of complexes containing β chimeras that included β4 TM domains mimicked cbv1+*wt*β4 responses. Support:R37-AA011560 (AMD).

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**The Action of the Neuroprotective Compound Riluzole on Kv Channels**

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The mechanism of action of the neuroprotective agent riluzole, clinically used against amyotrophic lateral sclerosis (ALS), is poorly understood. Studies have indicated multiple effects on several ion channel types. For instance, Kv1.5 and Kv3.1 have been reported to be blocked in the closed and inactivated states. In the present study the effects on Kv1.1, Kv1.3 and Kv2.1 channels expressed in Xenopus oocytes were studied, using the two-electrode voltage-clamp system. Riluzole reversibly inhibited the channels in a concentration dependent manner with IC50 values for fully open channels of 250, 150 and 700 mikroM, respectively. The slow inactivation of Kv1.1 was accelerated and the steady state inactivation and the peak activation curves were shifted to the left. However, the corresponding curves for Kv2.1 did not show shifts. The preliminary results of an kinetic analysis suggest that riluzole blocks both Kv1 channels in the open state, while the Kv2.1 channel was blocked both in the open and a closed state. The results do not show a block of inactivated channels, thus suggesting that the riluzole effects on Kv1.1, Kv1.3 and Kv2.1 may deviate in several respects from those on Kv1.5 and Kv3.1.

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**Integrated Structural Biology of the KCNQ1-KCNE3 Complex**

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KCNQ1 is a voltage-gated potassium channel with key physiological roles in cardiac repolarization and intestinal and tracheal ion transport. KCNE3 is a single span integral membrane β subunit that is co-expressed with KCNQ1 in a number of tissues. KCNE3 is required for KCNQ1 trafficking and functional modulation of, KCNQ1 in the basolateral membranes of intestinal and tracheal epithelia. This study utilizes an integreted functional and structural approach to probe the mechanism of KCNE3 modulation of KCNQ1. Whole cell patch clamp electrophysiology of KCNE3 and KCNE3 mutants with various KCNQ1 and KCNQ4 chimeras give insight into the functional determinants of modulation. Solution NMR is used to determine the structure of KCNE3 in isotropic bicelles. The functional and structural data are then coupled with comparative models of the KCNQ1 channel and computational techniques to generate structural models of the KCNQ1-KCNE3 complex. These atomically explicit structural models have predictive ability and yet are readily refined with addition of new functional and/or structural data. This work was supported by US NIH grant RO1 DC007416.

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**The Distal End of Carboxyl-Terminus is Not Essential for the Assembly of Rat Eag1 Potassium Channels**

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The assembly of four pore-forming α-subunits into tetramers is a prerequisite for the formation of functional $K^+$ channels. A short carboxyl assembly domain (CAD) in the distal end of the cytoplasmic carboxyl-terminus has been implicated in the assembly of Eag α-subunits, a subfamily of the ether-à-go-go $K^+$ channel family. The precise role of CAD in the formation of Eag tetrameric channels, however, remains unclear. Moreover, it has not been determined whether other protein regions also contribute to the assembly of Eag subunits. We addressed these questions by studying the biophysical properties of a series of different rat Eag1 (rEag1) truncation mutants. Two truncation mutants without CAD (K848X & W823X) yielded functional phenotypes similar to those for wild-type (WT) rEag1 channels. Furthermore, non-functional rEag1 truncation mutants lacking the distal region of the carboxyl-terminus displayed substantial dominant-negative effects on the functional expression of WT as well as K848X and W823X channels. Our co-immunoprecipitation studies further revealed that truncation mutants containing no CAD indeed displayed significant association with rEag1-WT subunits. Finally, surface biotinylation and protein glycosylation analyses demonstrated that progressive truncations of the carboxyl-terminus resulted in aggravating disruptions of membrane trafficking and glycosylation of rEag1 proteins. Overall, our data suggest that the distal carboxyl-terminus, including CAD, is dispensable for the assembly of rEag1 $K^+$ channels, but may instead be essential for ensuring proper protein biosynthesis. We propose that the S6 segment and the proximal carboxyl-terminus may constitute the principal subunit recognition site for the assembly of rEag1 channels.

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**14-3-3 is the Binding Partner of the Voltage-Gated Eag Potassium Channel**

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The ether-á-go-go (Eag) potassium ($K^+$) channel belongs to the superfamily of voltage-gated $K^+$ channel and is widely expressed in various regions of the brain. In the rat brain, there are two isoforms of Eag proteins, rat Eag1 (rEag1) and rat Eag2 (rEag2), sharing a high sequence homology. Despite their abundance in the brain, the precise neurophysiological significance of Eag channels remains unclear. In order to gain insight into the function of rEag1 channels in the brain, we have used yeast two-hybrid screening to identify rEag1-interacting proteins from the rat brain cDNA library. One of the clones we identified is 14-3-3, which belongs to a small acidic protein family and is abundantly expressed in the brain. We have performed the co-immunoprecipitation, GST pull-down assay, and immunofluorescence staining to confirm the interactions between rEag1 and 14-3-3. Furthermore, we demonstrated that 14-3-3 reduced the functional expression of rEag1 $K^+$ currents but